Agonist-induced desensitization and phosphorylation of m1-muscarinic receptors

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Pre-stimulation of Chinese hamster ovary (CHO) cells expressing the human m1-muscarinic receptor (CHO-m1 cells) with a maximally effective concentration of the muscarinic agonist methacholine resulted in desensitization of Ins(1,4,5)P_3 accumulation, apparent as a $\sim$ 4-fold shift in the agonist dose–response curve. Agonist-induced desensitization was rapid (detectable by 10 s) and concentration dependent ($EC_{50} = 8.2 \pm 2.2\ \mu M$) and resulted in a complete loss of receptor reserve for the agonist-stimulated Ins(1,4,5)P_3 response. An investigation of the possible mechanisms involved in m1-muscarinic receptor desensitization indicated that agonist-induced receptor internalization, PtdIns(4,5)P_2 depletion or an increased rate of Ins(1,4,5)P_3 metabolism were not involved. m1-Muscarinic receptors did, however, undergo rapid agonist-induced phosphorylation with a time course that was consistent with an involvement in receptor desensitization. Characterization studies indicated that the receptor-specific kinase involved was distinct from protein kinase C and other second-messenger-dependent protein kinases. Since previous studies have suggested that the m3-muscarinic receptor subtype undergoes agonist-dependent phosphorylation via casein kinase 1α (CK1α) [Tobin, Totty, Sterlin and Nahorski (1997) J. Biol. Chem. **272**, 20844–20849], we examined the ability of m1-muscarinic receptors to be phosphorylated by this kinase. In reconstitution experiments, CK1α was able to phosphorylate purified, soluble m1-muscarinic receptors in an agonist-dependent manner.

Key words: G-proteins, pharmacology, protein phosphorylation.

INTRODUCTION

Agonist-sensitive phosphorylation of G-protein-coupled receptors (GPCRs) is a general regulatory mechanism employed by nearly all GPCRs [1–4]. In the case of the β-adrenoceptor, phosphorylation at sites on the C-terminal tail and third intracellular loop of the receptor by protein kinase A and the receptor-specific kinase, β-adrenergic receptor kinase (GRK-2), mediates rapid uncoupling of the β-adrenoceptor from its G-protein [5]. Further studies, initially on the cyclase-linked m2-muscarinic receptor [6] and more recently on phospholipase C (PLC)-coupled receptors (for example [7]), have suggested that GRK-2 and related protein kinases that make up the G-protein-coupled receptor kinase (GRK) family [8] are involved in the agonist-driven phosphorylation of a large number of GPCRs (for reviews see [1–4,8,9])

An association between diminished PLC signalling and receptor phosphorylation has been established, for example with the m3-muscarinic receptor, where rapid agonist-mediated receptor phosphorylation correlates temporally with receptor desensitization [10–12], and the α_{1B}-adrenoceptor, where C-terminal receptor mutants, which were unable to undergo agonist-dependent receptor phosphorylation, also failed to exhibit agonist-dependent desensitization of Ins(1,4,5)P_3 generation [13]. Furthermore, co-transfection studies of the α_{1B}-adrenergic receptor with GRK-2, GRK-6 and a GRK-2 dominant-negative mutant have indicated that the receptor is phosphorylated in an agonist-dependent manner by at least one member of the GRK family [14]. Similar co-transfection studies or reconstitution studies have implicated GRK-2 in the phosphorylation of the PLC-coupled substance P [7], angiotensin II type 1_A [15], bradykinin B_2 [16] and endothelin [17] receptors. These studies lend weight to the growing body of evidence linking PLC-coupled receptor phosphorylation, potentially via GRKs, to receptor desensitization.

However, recent studies from our laboratory have demonstrated the existence of an alternative protein kinase pathway from that of the GRKs for the phosphorylation of GPCRs. A 40 kDa protein kinase, purified from porcine cerebellum, was found to phosphorylate a bacterial fusion protein containing the third intracellular loop of the PLC-coupled m3-muscarinic receptor [18]. This kinase, called muscarinic receptor kinase, was shown to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner [18]. Subsequent amino acid sequence analysis has identified the muscarinic receptor kinase as casein kinase 1α (CK1α) [19]. Studies on purified recombinant kinase confirmed that CK1α was able to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner [19]. Furthermore, CK1α was able to phosphorylate rhodopsin in a stimulus-dependent manner, suggesting that CK1α offers an alternative protein kinase pathway from that of the GRKs for the phosphorylation of GPCRs [19].

Agonist-stimulation of the m3-muscarinic receptor expressed in Chinese hamster ovary (CHO) cells results in a rapid elevation

Abbreviations used: CK1α, casein kinase 1α; CHO, Chinese hamster ovary; CHO-m1 cells, CHO cells expressing the human m1-muscarinic receptor; GRK, G-protein coupled receptor kinase, GPCR, G-protein-coupled receptor; PLC, phospholipase C; NMS, *N*-methylscopolamine; PKC, protein kinase C; K_d, equilibrium dissociation constant; GRK-2, β-adrenergic receptor kinase; ABT, aminobenztropine; PrBCM, propylbenzilylcholine mustard.

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in Ins(1,4,5)P_3 that peaks after 5–10 s and falls to a sustained plateau phase that is maintained for tens of minutes [10]. This response undergoes desensitization following a brief period of agonist pre-exposure; a phenomenon that correlates temporally with agonist-sensitive receptor phosphorylation, which occurs within seconds of agonist addition [10,11]. In the present study, we examine whether the other predominant-PLC-coupled muscarinic receptor subtype, namely the m1-muscarinic receptor, is regulated in a similar manner. Particularly as preliminary studies have shown that both muscarinic receptor subtypes have a similar temporal pattern of inositol phosphate generation [20,21]. We report, in the present work, that m1-muscarinic receptors are able to undergo rapid agonist-induced desensitization and phosphorylation and, by using reconstitution experiments with soluble purified receptors, that CK1α has the potential to mediate agonist-sensitive m1-muscarinic receptor phosphorylation.

MATERIALS AND METHODS

Materials

[^{3}H]Ins(1,4,5)P_3 (17–20 Ci/mmol) was obtained from NEN–Dupont. [^{3}H]Inositol (20 Ci/mmol), [^{32}P]P_i (10 mCi/mmol) and *N*-[^{3}H]methylscopolamine ([^{3}H]NMS; 80 Ci/mmol) were purchased from Amersham International. Cell-culture reagents were obtained from Gibco. Ro-318220 was a gift from Roche Ltd. (Welwyn Garden City, Herts, U.K.). Centricon 50 was from Amicon; the hydroxyapatite column was from Bio-Rad; pVL1392 and BaculoGold system were from Pharmingen. Aminobenztropine (ABT) was from Research Biochemicals International and Sepharose 4B was from Pharmacia. All other materials were from Sigma.

Cell culture

CHO cells expressing m1-muscarinic receptors (CHO-m1 cells) were a gift from Dr. N. J. Buckley (Department of Pharmacology, University College London, London, U.K.). Cells were maintained as described previously [10]. The insect cell line, *Spodoptera frugiperda* (Sf9), was maintained in serum-free medium (Sf-900 II SFM) at 27 °C, either in monolayer or in shaker flasks agitated by orbital rotation at 130 rev./min.

Ins(1,4,5)P_3 mass determination

Plated-out CHO-m1 cells, grown in 24-well dishes, were washed with Krebs/Hepes buffer, pH 7.4 (4.2 mM $NaHCO_3$/118 mM NaCl/4.7 mM KCl/2 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/10 mM glucose/10 mM Hepes). To initiate the experiments the Krebs/Hepes buffer was removed and replaced with Krebs/Hepes buffer containing drugs at the appropriate concentrations. All stimulations were carried out at 37 °C. Reactions were terminated by the addition of an equal volume of trichloroacetic acid (1 M). Extraction and assay of Ins(1,4,5)P_3 has been described previously [10,22]. In desensitization experiments, cells were challenged with methacholine for a given time, were rapidly washed three times with warm Krebs/Hepes buffer and incubated for 3–5 min. The cells were then re-stimulated and Ins(1,4,5)P_3 accumulation was determined.

Agonist binding studies

Agonist binding to intact CHO-m1 cells was assessed by inhibition of [^{3}H]NMS binding to cells incubated overnight at 4 °C to prevent agonist-induced internalization of the receptors. After incubation, the cells were placed on ice and washed three times with ice-cold Krebs/Hepes buffer, pH 7.4, in order to separate bound from free [^{3}H]NMS. Cells were then solubilized with RIPA solubilization buffer [10 mM Tris base/10 mM EDTA/500 mM NaCl/1% (v/v) Nonidet N-40/0.5% (w/v) SDS, pH 7.4] and the radioactivity in the cell extract was estimated by scintillation spectroscopy. In experiments to determine the effect of prestimulation on the equilibrium dissociation constant (K_d) for agonist binding, cells were stimulated for 5 min at 37 °C then placed immediately on ice before being washed rapidly four times with ice-cold Krebs/Hepes buffer, to prevent internalization of receptors. The binding of [^{3}H]NMS to intact cells revealed a K_d of 0.43 ± 0.1 nM ($n = 3$) and a B_{max} of 1.6 ± 0.4 pmol/mg protein ($n = 3$).

Inositol phospholipid extraction and separation

All experiments were carried out at 37 °C in Krebs/Hepes (pH 7.4) buffer supplemented with 10 mM inositol. CHO-m1 cells, grown in 24-well dishes, were labelled with [^{3}H]inositol for 48 h. Cells were stimulated and the reaction was terminated as described above for Ins(1,4,5)P_3 determinations. The acidified medium was removed and the cell pellet was washed with 1 ml of 5% (v/v) perchloric acid/1 mM EDTA followed by 1 ml of water. Lipids were extracted by the addition of 0.94 ml of chloroform/methanol/12 M HCl (40:80:1, by vol.) for 15 min at room temperature, followed by the addition of 0.31 ml of chloroform and 0.56 ml of 0.1 M HCl, to achieve phase partition. A sample of the lower phase (400 μl) was removed, dried and stored under N_2 for subsequent processing.

For deacylation of [^{3}H]polyphosphoinositides, dried lipid samples were dissolved in 1.2 ml of chloroform/methanol (5:1, v/v) and 0.4 ml of 0.5 M NaOH in methanol/water (19:1, v/v) was added and the samples were thoroughly mixed, incubated at room temperature for 20 min and centrifuged (3000 ***g*** for 10 min). A sample of the upper phase (1 ml), which contained the [^{3}H]glycerophosphoinositol (phosphates) as deacylation products, was recovered and neutralized by passage through a Dowex-50 (H^+ form) column (1 ml bed volume). The column was washed twice with 1 ml of water and the pooled eluate was adjusted to pH 7.4 by the addition of $NaHCO_3$ before addition to a Dowex (AG1-x8), formate anion-exchange column. The [^{3}H]glycerophosphoinositol (phosphates) were eluted as described previously [21]. The presence of [^{3}H]glycerophosphoinositol, [^{3}H]glycero-PtdIns4*P* and [^{3}H]glyceroPtdIns(4,5)P_2 are indices for phosphatidylinositol, PtdIns4*P* and PtdIns(4,5)P_2 respectively.

Agonist-induced internalization of m1-muscarinic receptors

Agonist-induced internalization of m1-muscarinic receptors was assessed by stimulating CHO-m1 cells with methacholine (1 mM) for set times at 37 °C. Cell monolayers were then placed on ice and washed rapidly four times in ice-cold Krebs/Hepes buffer, pH 7.4. The proportion of cell surface muscarinic receptors remaining was determined by the degree of binding after incubation in a saturating concentration of [^{3}H]NMS at 4 °C overnight.

Immunization and m1-antiserum production

An antiserum designated Mc-1 was raised against a peptide corresponding to the last 20 amino acids of the C-terminal domain of the human m1-muscarinic receptor (RWRKIPKRP GSVHRTPSRQC). This peptide was conjugated to keyhole-limpet haemocyanin and was used to immunize New Zealand

White rabbits. Characterization of the antiserum employed Western blots derived from membranes prepared from CHO cells expressing m1-, m2-, m3-, m4- and m5-muscarinic receptors. Standard Western-blotting procedures were employed, essentially as described previously [11]. The Mc-1 antiserum was used in a 1:500 dilution.

In vivo ^{32}P-labelling and immunoprecipitation

The *in vivo* labelling of ATP pools in CHO cells, agonist-stimulation, receptor solubilization and immunoprecipitation were carried out as described previously for the m3-muscarinic receptor [11]. Immunoprecipitation of m1- and m3-muscarinic receptors was carried out using a 1:500 dilution of antiserum Mc-1 and the m3-muscarinic-specific antiserum Ab332 [11] respectively.

Immunoprecipitation of [^{3}H]propylbenzilylcholine mustard ([^{3}H]PrBCM)

CHO-m1 cells were harvested and resuspended in Krebs/Hepes buffer (pH 7.4) to a concentration of 1×10^6 cells/ml. To 1 ml of cell suspension a saturating concentration of [^{3}H]PrBCM (10 nM) was added for 2 h (37 °C). Non-specific binding was determined in the presence of 2 μM atropine. Labelling was terminated by pelleting the cells by centrifugation at 3000 ***g*** for 3 min. The cell pellet was resuspended in 1 ml of solubilization buffer and the m1-muscarinic receptor was immunoprecipitated from the solubilized sample as described above. The immunoprecipitated proteins were separated by SDS/PAGE (10 % gel) The gels were then impregnated with Amplify (Amersham), fixed, dried and autoradiographed.

Purification of CK1α kinase from porcine brain

The procedure used for purification of CK1α from porcine cerebellum has been described previously [18].

Preparation of m1-muscarinic receptor expressing baculovirus

cDNA encoding the human m1-muscarinic receptor (a gift from Dr. J. Silvio Gutkind, National Institute for Dental Research, National Institutes of Health, Bethesda, MD, U.S.A.) was subcloned into the baculovirus transfer vector, pVL1392, at the *Bgl*II site to generate the pVL1392/M1 plasmid. Recombinant baculovirus was then generated using the BaculoGold system.

Purification of recombinant m1-muscarinic receptors

A culture (1 litre) of Sf9 cells at 1.4×10^6 cells/ml was infected at a multiplicity of infection of 5 with recombinant m1 baculovirus. Two days after infection, cells were harvested by centrifugation at 1500 ***g*** for 10 min and resuspended at 10^7 cells/ml in buffer A (20 mM Tris/HCl, pH 7.4/1 mM EDTA/4 mM Pefabloc/2 μM leupeptin/0.3 μM aprotinin/1.4 μM pepstatin) and disrupted, on ice, in a Dounce homogenizer. The homogenate was centrifuged at 40000 ***g*** for 15 min at 4 °C, and resuspended in one-quarter of the original volume in buffer B (buffer A without EDTA). Dounce homogenization and centrifugation were repeated as above, the final pellet was resuspended in buffer B at a protein concentration of 2 mg/ml and stored at −85 °C.

Receptor solubilization and purification were carried out essentially as described by Haga and Haga [23]. ABT was coupled to Sepharose 4B as described previously [24]. The ABT content of the affinity resin was 1.75 mol/ml. All procedures were carried out at 4 °C. The receptor was extracted by mixing membranes with solubilization buffer [20 mM potassium phosphate buffer, pH 7.0/100 mM NaCl/1 mM EDTA/0.1 % (w/v) sodium cholate/1 % (w/v) digitonin] for 1 h at 4 °C, with stirring. The mixture was centrifuged at 175000 ***g*** for 30 min and the supernatant (90 ml) was immediately loaded on to an ABT-agarose column (10 ml bed volume, 0.5 ml/min) that had been equilibrated with 20 mM potassium phosphate buffer, pH 7.0/0.1 % (w/v) digitonin. The ABT column was washed overnight with 100 ml of washing solution (20 mM potassium phosphate buffer, pH 7.0/0.1 % digitonin/150 mM NaCl). A hydroxyapatite column (0.5-ml bed volume) was connected, in series, to the ABT-agarose column and the receptor was eluted from the ABT-agarose column with 50 ml of washing solution that included 0.1 mM atropine sulphate. The hydroxyapatite column was then disconnected from the ABT column and receptors were eluted from the hydroxyapatite with increasing concentrations of potassium phosphate buffer, pH 7.0, [solution A, 100 mM (5 ml); solution B, 500 mM (1.5 ml); followed by solution C, 900 mM (1.5 ml)] in 0.1 % (w/v) digitonin/1 mM carbachol.

The soluble receptor was quantified using a binding assay as described previously [23,25]. The yield of receptor-binding sites extracted from the membranes was 410 pmol (62 %), 38 % of the binding sites that were applied to the ABT column were lost in the flow-through and washing procedures and 8.5 % of the receptor was recovered in the eluates from the hydroxyapatite column with solutions B and C; most of the binding sites were recovered with solution C. The receptor was concentrated by centrifugal ultrafiltration (Centricon 50) to 0.1–0.4 pmol/μl, divided into portions and stored at −85 °C. Receptor recovery after concentration was > 80 %. The specific activity of the purified receptor preparation was 5 nmol/mg protein.

Phosphorylation of purified m1-muscarinic receptors

Phosphorylation of purified m1-muscarinic receptors, solubilized in 0.05 % digitonin, was carried out in (25 μl final volume) kinase buffer [20 mM Tris/HCl, pH 7.4/10 mM $MgCl_2$/1 mM EGTA/2 mM dithiothreitol/0.05 % (w/v) digitonin] containing 0.2 pmol of m1-muscarinic receptor and 0.25 pmol of purified CK1α; controls were buffer alone. After incubation for 60 min at 30 °C, the reaction was terminated by the addition of 10 μl of 0.2 % (w/v) BSA/5 % (w/v) charcoal in digitonin/TE buffer [10 mM Tris base, pH 7.4/10 mM EDTA/0.05 % (w/v) digitonin]. The sample was then briefly centrifuged in a microfuge and the supernatant was subjected to SDS/PAGE (10 % gel); an autoradiogram was obtained from the gel.

Data analysis

Data were analysed using GraphPad Prism software and are presented as the mean ± S.E.M. of at least three separate determinations. Statistical significance was set at $P < 0.05$ using unpaired Student's t test analysis. Analysis of autoradiograms was by laser densitometry (Ultrascan XL1; LKB)

RESULTS

m1-Muscarinic receptor-mediated Ins(1,4,5)P_3 response and desensitization

We have reported previously that, in CHO-m1 cells, the Ins(1,4,5)P_3 response shows a peak at 10 s after agonist stimulation followed by a sustained phase which continues for tens of minutes [20,21]. This peak/plateau Ins(1,4,5)P_3 response is seen in a number of PLC-coupled receptors, including the

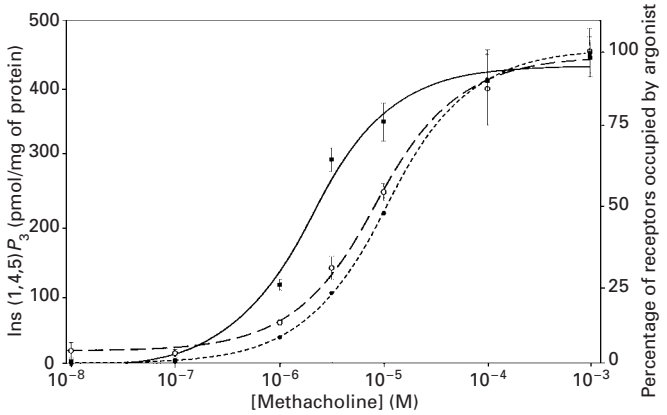

Figure 1 Desensitization of methacholine-stimulated Ins(1,4,5)P_3 response

Dose–responses for agonist-induced Ins(1,4,5)P_3 production at 10 s for control CHO-m1 cells (■; continuous line) and CHO-m1 cells that had been pretreated with methacholine (1 mM) for 5 min (○). Results represent means ± S.E.M. of four separate determinations. Also shown is the agonist-binding curve (●) calculated for each concentration of methacholine (as described in the Results section), using 11.2 μM as the dissociation constant for methacholine binding to CHO-m1 cells.

m3-muscarinic receptor subtype [10]. Previous studies on the m3-muscarinic receptor demonstrated that the early peak phase of the inositol phosphate response is sensitive to desensitization resulting from a short 5 min pre-exposure to agonist [10]. A similar method was employed in the present study to investigate the possibility that the m1-muscarinic receptor underwent desensitization. Pre-exposure of CHO-m1 cells to the full agonist methacholine (1 mM) for 5 min resulted in desensitization of the peak (10 s) Ins(1,4,5)P_3 response. The overall effect of desensitization was apparent as a rightward shift in the methacholine dose–response curve, where the EC_{50} was increased from 2.4 ± 0.8 μM ($n = 4$) for the control response to 8.6 ± 1.7 μM ($n = 4$) for the desensitized response (Figure 1).

A comparison was made between the desensitized dose–response curve and the methacholine binding curve with the m1-muscarinic receptor. As can be seen in Figure 1, following desensitization, the dose–response curve for the Ins(1,4,5)P_3 response overlies the agonist-occupation curve (the EC_{50} for the desensitized methacholine response was not statistically different from the apparent K_d obtained for methacholine binding to the m1-muscarinic receptor; $K_d = 11.7 \pm 2.2$ μM, $n = 4$). By contrast, the control Ins(1,4,5)P_3 dose–response curve lies to the right of the agonist-occupation curve. These data indicate that there is a receptor reserve for the methacholine Ins(1,4,5)P_3 response, which is completely lost following desensitization and demonstrate the importance of analysis of a full dose–response curve (or at least a submaximal response) when investigating desensitization which may result in a decrease in the efficacy of an agonist without any change in its maximal response. A pre-challenge with 1 mM methacholine as the desensitizing stimulus was used in subsequent experiments, followed by a challenge with a submaximal (3.3 μM) concentration of methacholine.

In time-course experiments, desensitization of the methacholine (3.3 μM) peak Ins(1,4,5)P_3 response was very rapid being apparent within 10 s of agonist addition and maximal by 60 s (Figure 2a). In addition, desensitization was highly dependent on the concentration of agonist employed during the 5 min pre-challenge with an EC_{50} for methacholine of 8.2 ± 2.2 μM.

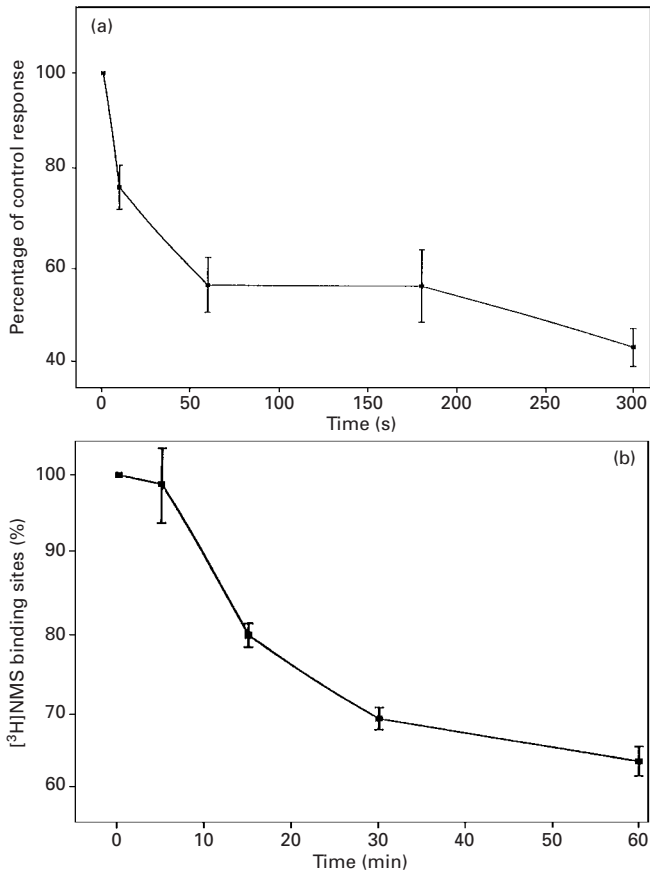

Figure 2 Time course for agonist-induced m1-muscarinic receptor internalization and desensitization

(**a**) Time course for agonist-induced desensitization of m1-muscarinic receptors. Plated-out CHO-m1 cells were stimulated with methacholine (1 mM) for various times. The cells were then washed three times with Krebs/Hepes buffer, followed by a recovery period after which the Ins(1,4,5)P_3 response to methacholine (3.3 μM) stimulation for 10 s was assessed. Results are shown as means ± S.E.M. for 4–6 separate determinations and expressed as percentage of control response to methacholine (3.3 μM). (**b**) Time course for m1-muscarinic receptor internalization. Plated-out CHO-m1 cells were stimulated with methacholine (1 mM) for the times indicated at 37 °C. Reactions were terminated by placing the cells on ice and washing four times with ice-cold Krebs/Hepes buffer. The number of cell surface receptors remaining was determined by the binding of [^{3}H]NMS overnight at 4 °C. Data presented are the means ± S.E.M. of three separate experiments.

m1-Muscarinic receptor internalization

In order to test whether receptor sequestration and internalization had a role to play in the desensitization response a time course for agonist-mediated receptor sequestration was studied. Figure 2(b) shows that there was no significant change in m1-muscarinic-receptor cell-surface number following a 5 min exposure to methacholine (1 mM), although by 60 min there was a 36 % decrease in the number of cell-surface receptors.

m1-Muscarinic-receptor-mediated phosphoinositide response and desensitization

Stimulation of CHO-m1 cells with a sub-maximal dose of methacholine (3.3 μM) resulted in a rapid fall in PtdIns(4,5)P_2, so that by 10 s levels had fallen by 48.8 ± 3.1 % ($n = 4$). Agonist pre-challenge (1 mM) resulted in a 43 % reduction in the methacholine-stimulated (3.3 μM) PtdIns(4,5)P_2 response at 10 s (Fig-

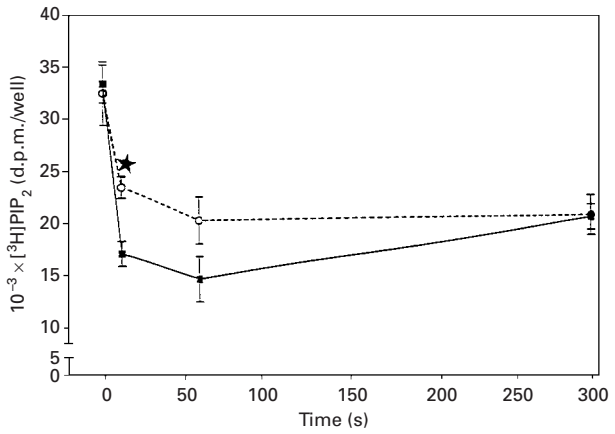

Figure 3 Agonist-induced depletion of [^{3}H]PtdIns(4,5)P_2

Following equilibrium labelling of CHO-m1 cells with [^{3}H]inositol, cells were either exposed to a 5 min pre-challenge with methacholine (○) or vehicle (■). The cells were then washed three times and the [^{3}H]PtdIns(4,5)P_2 pools were analysed following stimulation with 3.3 μM methacholine for the times indicated. Reactions were terminated by the addition of an equal volume of trichloroacetic acid (1 M). Extraction, deacylation and separation of [^{3}H]PtdIns(4,5)P_2 were carried out as described in the Materials and methods section. Data are presented as the means ± S.E.M. of 4–6 independent determinations; * indicates a significant difference ($P < 0.01$) between the control and pre-stimulation responses.

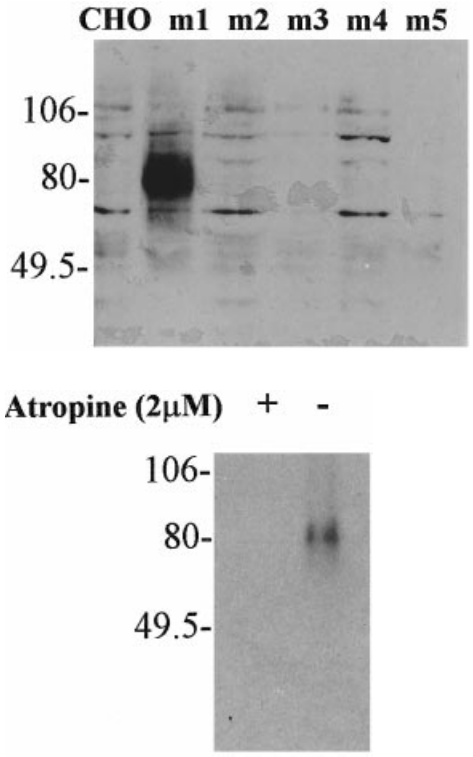

Figure 4 Characterization of the m1-muscarinic receptor-specific antiserum, Mc-1

Upper panel: Western blot using Mc-1 (1:500 dilution) to probe a nitrocellulose membrane containing membrane proteins (20 μg of protein per lane) from either CHO cells or CHO cells expressing m1, m2, m3, m4 or m5 muscarinic receptors. Lower panel: autoradiogram of [^{3}H]PrBCM-labelled m1-muscarinic receptors immunoprecipitated with Mc-1 antiserum. CHO-m1 cells were labelled with 10 nM [^{3}H]PrBCM for 2 h in the presence or absence of atropine (2 μM). The cells were solubilized and the receptors immunoprecipitated using Mc-1 (1:500 dilution) as described in the Materials and methods section. Positions of molecular-mass markers (kDa) are shown on the left.

ure 3). It is important to note that the wash period of 3 min between the first and second application of agonist was sufficient to allow PtdIns(4,5)P_2 levels to completely recover (compare points at time zero, Figure 3). Therefore the reduced PtdIns(4,5)P_2 response following an agonist pre-challenge was not due to a depletion of PtdIns(4,5)P_2 pools. These data are consistent with those of the Ins(1,4,5)P_3 data and suggest that there is a rapid desensitization of the m1-muscarinic receptor signalling at the level of receptor–PLC coupling.

m1-Muscarinic receptor phosphorylation

Accumulating evidence in the literature has suggested that second-messenger production mediated through many G-protein-linked receptors might be acutely regulated through agonist-sensitive phosphorylation at the receptor level [1–4,8,9]. It seemed appropriate, therefore, to examine the possibility that the m1-muscarinic receptor might also undergo agonist-dependent phosphorylation. An antiserum (Mc-1) was raised to the C-terminal tail of the m1-muscarinic receptor and, on immunoblots, the receptor was revealed as a diffuse band at ~ 80 kDa (Figure 4, upper panel). The antiserum did not cross-react with membrane preparations from non-transfected CHO cells or CHO cells expressing either the m2-, m3-, m4- or m5-muscarinic receptor subtypes (Figure 4, upper panel). m1-Muscarinic receptors radiolabelled with the irreversible muscarinic antagonist [^{3}H]PrBCM were also specifically immunoprecipitated by the Mc-1 antiserum (Figure 4, lower panel)

In CHO-m1 cells labelled with [^{32}P]P_i, immunoprecipitation of m1-muscarinic receptors revealed that the receptor was phosphorylated under basal conditions (Figure 5). Addition of methacholine (1 mM) for 15 min resulted in a ~ 4-fold increase in receptor phosphorylation for the 80 kDa m1-muscarinic receptor. This was comparable with the ~ 5-fold increase in phosphorylation observed for the 100 kDa m3-muscarinic receptor immunoprecipitated with an m3-muscarinic-receptor-

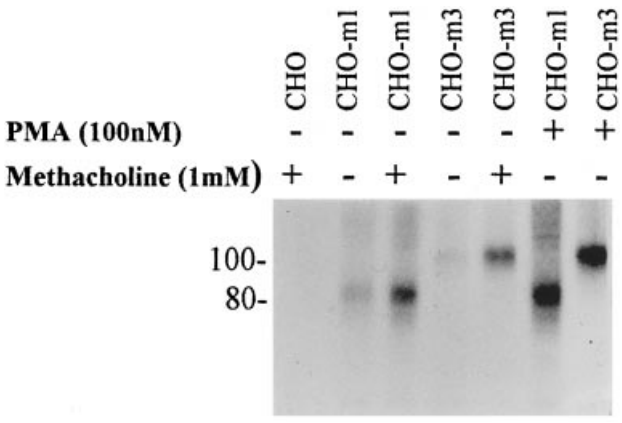

Figure 5 Phosphorylation of m1- and m3-muscarinic receptors

Cell suspensions were labelled with [^{32}P]P_i for 1 h and then stimulated with either methacholine (1 mM) or PMA (100 nM) for 15 min. Reactions were terminated by the addition of ice-cold RIPA solubilization buffer and receptors were immunoprecipitated using subtype-specific antiserum (see the Materials and methods section). Positions of molecular-mass markers (kDa) are shown on the left.

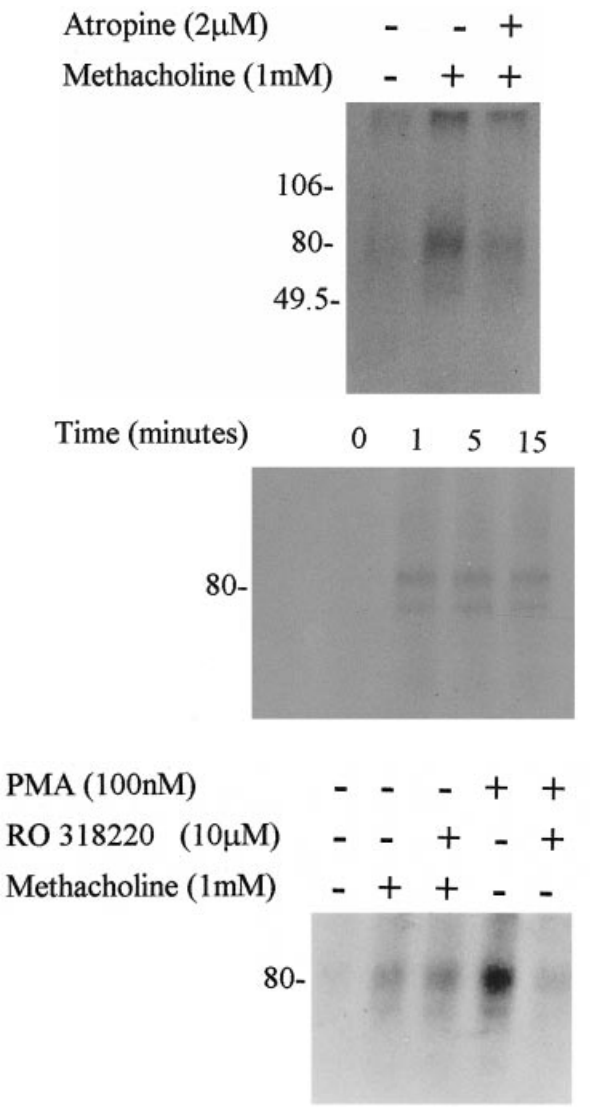

Figure 6 Characterization of m1-muscarinic receptor phosphorylation

Upper panel: inhibition of methacholine-induced phosphorylation of m1-muscarinic receptor by the muscarinic receptor antagonist atropine (2 μM). Middle panel: time course for phosphorylation of the m1-muscarinic receptor following addition of 1 mM methacholine. Lower panel: effect of PKC inhibition with RO-318220 (10 μM) on agonist- and PKC-mediated m1-muscarinic receptor phosphorylation. All stimulations were for 15 min. The results shown are representative of 2–3 experiments. Positions of molecular-mass markers (kDa) are shown on the left.

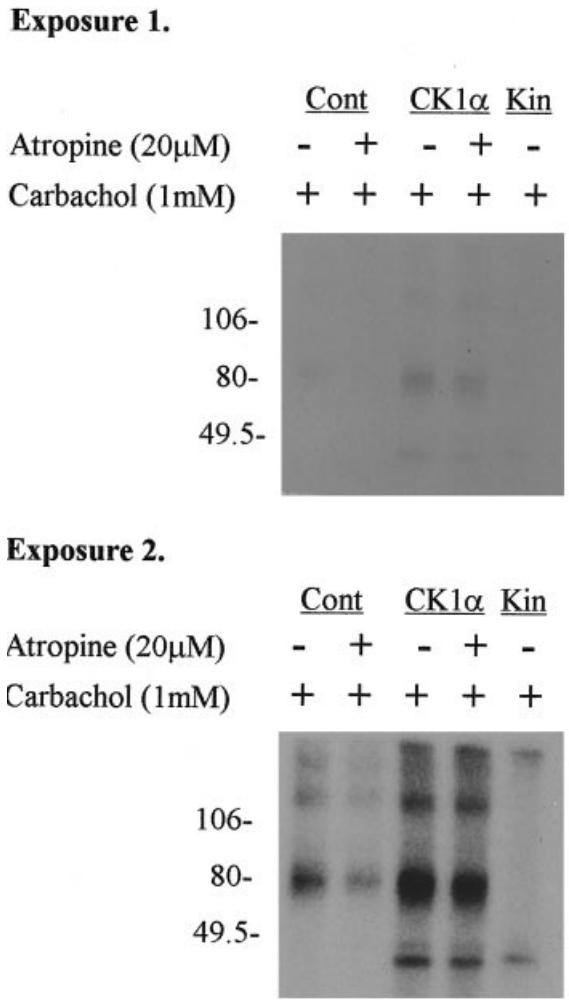

Figure 7 *In vitro* phosphorylation of purified m1-muscarinic receptors by CK1α

Purified m1-muscarinic receptors (0.2 pmol) were incubated with CK1α (0.25 pmol) purified from porcine cerebellum (lanes marked CK1α) or with buffer alone (cont). A reaction mixture containing CK1α alone was also included (Kin). The reaction was continued for 60 min at 30 °C in the presence of carbachol (1 mM) or carbachol plus atropine (20 μM). The reaction was stopped by removing excess nucleotides with a charcoal/BSA slurry and the proteins were resolved by SDS/PAGE (10% gel). Exposure 1: the gel was exposed overnight to Hyperfilm-MP (Amersham); exposure 2: the gel was exposed overnight to high-sensitivity BioMax-MS film (Kodak). The results are representative of four experiments. Positions of molecular-mass markers (kDa) are shown on the left.

specific antiserum (Figure 5). Methacholine-induced phosphorylation of the m1-muscarinic receptor was inhibited by pre-incubation with atropine, a muscarinic antagonist (Figure 6, upper panel). Time-course studies revealed that the m1-muscarinic receptor was rapidly phosphorylated following addition of agonist, with phosphorylation being maximal by 1 min (Figure 6, middle panel)

Activation of protein kinase C (PKC) by the phorbol ester PMA (100 nM) also resulted in an increase in m1-muscarinic receptor phosphorylation in a similar manner to that seen for the m3-muscarinic receptor ($\sim$ 7- and $\sim$ 10-fold increases in phosphorylation respectively) (Figure 5). This suggested that the m1-muscarinic receptor could be phosphorylated by PKC. Whether PKC was the kinase that mediated agonist-induced phosphorylation was investigated by testing the susceptibility of agonist-stimulated phosphorylation to inhibition by Ro-318220, a specific inhibitor of PKC [26]. Methacholine-stimulated m1-muscarinic receptor phosphorylation was not blocked by a 15 min pre-incubation with Ro-318220 in contrast to the PMA-induced effect, which was completely abolished by pre-treatment with this inhibitor (Figure 6, lower panel). In addition, we found that elevation of either intracellular cAMP levels with forskolin (1 μM) or Ca^{2+} with ionomycin (1 μM) did not affect the phosphorylation state of m1-muscarinic receptors (results not shown). These data suggest that agonist-specific m1-muscarinic receptor phosphorylation was not due to the action of protein kinase A or Ca^{2+}/calmodulin-dependent protein kinase, or to the activity of phorbol-ester-activated PKC.

Phosphorylation of purified m1-muscarinic receptors by CK1α

The characteristics, described above, of the receptor kinase responsible for m1-muscarinic receptor phosphorylation in CHO cells are remarkably similar to those of the kinase responsible for m3-muscarinic receptor phosphorylation [11]. Detailed studies have suggested that the kinase involved in m3-muscarinic receptor phosphorylation is CK1α [18,19]. It therefore seemed appropriate to test whether CK1α had the potential to phosphorylate the m1-muscarinic receptor in an agonist-dependent fashion. This was investigated by testing the ability of purified, recombinant CK1α to phosphorylate the purified m1-muscarinic receptor solubilized in digitonin.

Solubilized m1-muscarinic receptors, purified from Sf9 cells expressing recombinant receptor, were reconstituted with CK1α purified from porcine cerebellum or with buffer alone. In order to maintain the stability of the solubilized m1-muscarinic receptor it was necessary to have the agonist carbachol present at all times. Thus basal phosphorylation was determined by addition of the antagonist atropine (20 μM). In both basal and stimulation experiments, carbachol was maintained at 1 mM.

As shown in Figure 7, there appears to be a receptor kinase that co-purifies with the m1-muscarinic receptor, since, under conditions where there is no added kinase (i.e. buffer alone), the m1-muscarinic receptor is phosphorylated in an agonist-dependent manner. In the absence of antagonist, phosphorylation by the co-purifying kinase was ~7-fold higher than in the presence of antagonist (compare lanes 1 and 2 in Figure 7).

Addition of CK1α purified from porcine brain results in an ~10-fold increase in basal receptor phosphorylation, which is further increased (by ~2-fold) following withdrawal of the antagonist (Figure 7). Also evident is the autophosphorylation of CK1α, which runs as a band at 40 kDa (Figure 7). Similar results have been obtained using recombinant CK1α purified from Sf9 cells (results not shown).

DISCUSSION

This study demonstrates that the m1-muscarinic receptor expressed in CHO cells undergoes desensitization following a short (10–300 s) agonist pre-exposure. Desensitization was apparent as a 4-fold shift in the agonist Ins(1,4,5)P_3 dose–response curve, eliminating the receptor reserve associated with the response without effecting the Ins(1,4,5)P_3 response to maximal agonist concentrations. In order to dissect the mechanism of desensitization we have investigated three possibilities: (i) the role of receptor internalization, (ii) PtdIns(4,5)P_2 pool depletion and (iii) receptor modification.

Previous studies have shown that muscarinic receptors can be internalized on agonist stimulation (e.g.[10,27–29]). The reduction in cell-surface receptor number induced by internalization could ultimately lead to a situation where the receptor reserve is abolished, leading to reduced responsiveness on agonist re-challenge [30]. In the present study, however, it was found that the onset of receptor internalization was too slow to account for the observed desensitization. No significant receptor internalization was observed 5 min after the addition of methacholine, a time point where receptor desensitization is maximal. However, agonist-induced receptor internalization was observed 15 min after the addition of agonist and by 60 min there was a 36% decrease in the number of cell-surface receptors present.

The possibility that agonist pre-stimulation depleted the PtdIns(4,5)P_2 substrate pool was also investigated. Previous studies have shown that muscarinic-receptor stimulation results in a rapid fall in PtdIns(4,5)P_2 levels [21,28,31]. These data were confirmed in the present study, where a submaximal stimulation of m1-muscarinic receptors resulted in a dramatic fall in PtdIns(4,5)P_2 levels. The hydrolysis of PtdIns(4,5)P_2 was attenuated by a pre-stimulation with a maximal concentration of agonist. Importantly, following pre-stimulation, PtdIns(4,5)P_2 levels recovered completely during the wash period. This indicated that the reduction in PtdIns(4,5)P_2 hydrolysis observed following agonist pre-challenge was not due to depletion of the PtdIns(4,5)P_2 pool. By the same argument, the desensitization of the Ins(1,4,5)P_3 response could not be due to PtdIns(4,5)P_2 pool depletion. These data also indicate that the desensitized Ins(1,4,5)P_3 response is not due to enhanced metabolism but reflects an uncoupling of the receptor from the PLC pathway, which most likely results in a stoichiometric decrease in PtdIns(4,5)P_2 hydrolysis.

Work over the last decade has established that agonist-sensitive phosphorylation of GPCRs probably plays a central regulatory role in transmembrane signalling [1–5,9]. Phosphorylation of PLC-coupled receptors is also thought to mediate receptor desensitization. This supposition is supported by studies that have found a correlation between functional desensitization and receptor phosphorylation in a number of PLC-coupled receptors, including the m3-muscarinic receptor [10–12], thrombin receptor [32], platelet activating factor receptor [33], neurokinin 2 receptor [34] and the α_{1B}-adrenoceptor [14]. Extensive studies on the adenylate-cyclase linked β-adrenoceptor and m2-muscarinic receptors have demonstrated that agonist-sensitive phosphorylation and desensitization is mediated by GRK-2 [5,9]. It is now clear however that GRK-2, and other members of the GRK family, can also phosphorylate PLC-coupled receptors [1,8], suggesting that these kinases have a broad range of receptor substrates that include adenylate cyclase and PLC-coupled receptors. However, studies from our laboratory have shown that the m3-muscarinic receptor is a substrate for a novel 40 kDa receptor kinase, previously called muscarinic receptor kinase but recently identified as CK1α [19]. Reconstitution studies have shown that CK1α is able to phosphorylate m3-muscarinic receptors and rhodopsin in a stimulus-dependent manner [19,35]. It appears, therefore, that there may be two independent routes of phosphorylation of PLC-coupled receptors, one via the GRKs and the other via CK1α.

Previous studies on m1-muscarinic receptors expressed in insect (*Sf* 9) cells suggested that this receptor subtype was not phosphorylated by agonist stimulation [36]. However, recent studies on purified m1-muscarinic receptors reconstituted with GRK-2 indicated that the receptor was capable of being phosphorylated in an agonist-sensitive fashion [37]. Using an m1-receptor-specific antiserum, it was shown in the present work, for the first time, that m1-muscarinic receptors are able to undergo agonist-induced phosphorylation in intact cells. Incubation with agonist resulted in a dramatic and rapid enhancement in the phosphorylation state of m1-muscarinic receptors in a manner akin to that reported previously for m3-muscarinic receptors [11]. Maximal phosphorylation was observed following a 1 min stimulation with agonist, which correlates with the rate of desensitization which was also maximal after 1 min. Both m1- and m3-muscarinic receptors also underwent agonist-independent phosphorylation in the presence of the PKC-activating phorbol ester, PMA. However, in common with previous findings with the m3-muscarinic receptor [11], we demonstrated that agonist-sensitive phosphorylation of m1-muscarinic receptors was not dependent on the activity of PKC since, unlike PMA-induced phosphorylation, agonist-stimulated phosphorylation was not inhibited by Ro-318220, a specific inhibitor of PKC [26]. This eliminated the possibility that a simple feedback process was in operation, where m1-muscarinic

receptor activation would have resulted in PLC-mediated diacylglycerol generation with subsequent activation of PKC and phosphorylation of the receptor.

m1-Muscarinic-receptor-mediated elevations in intracellular Ca^{2+} and cAMP have been reported previously [20,38]. However, in the present work, no enhancement of m1-muscarinic receptor phosphorylation was shown in instances where intracellular levels of cAMP and Ca^{2+} were raised independently of receptor activation by either forskolin, an activator of adenylate cyclase, or ionomycin, a Ca^{2+} ionophore (results not shown). The apparent inability of these agents to enhance the phosphorylation state of m1-muscarinic receptors makes it unlikely that protein kinase A or Ca^{2+}-calmodulin-dependent kinases are involved in agonist-dependent m1-muscarinic receptor phosphorylation.

The characteristics of m1-muscarinic-receptor phosphorylation in CHO cells appear similar to those previously reported for m3-muscarinic-receptor phosphorylation [11]. These characteristics include the rapid nature of agonist-mediated phosphorylation and the fact that the kinase responsible is distinct from PKC and other second messenger-regulated protein kinases [11]. Since previous studies have indicated that the cellular receptor kinase responsible for m3-muscarinic receptor phosphorylation is likely to be CK1α [19], the possibility that CK1α could also phosphorylate the m1-muscarinic receptor was examined. In the case of the m3-muscarinic receptor, because of the technical difficulties in purifying the receptor, it was not possible to reconstitute purified m3-muscarinic receptor with purified kinase. Instead, a membrane preparation from CHO cells had to be used as a source of receptor in the reconstitution studies, which demonstrated that CK1α could phosphorylate the receptor in an agonist-sensitive-manner [19]. In the present study, we were able to circumvent the potential problems inherent in a membrane preparation (e.g. contaminating kinases and consumption of ATP by membrane-associated ATPases) by using purified m1-muscarinic receptors. Reconstitution of purified digitonin-solubilized m1-muscarinic receptors with purified CK1α dramatically increased basal phosphorylation of the m1-muscarinic receptor. In the absence of antagonist (and presence of agonist), m1-muscarinic receptor phosphorylation was further increased demonstrating that the m1-muscarinic receptor is able to be phosphorylated by CK1α in an agonist-dependent manner. These *in vitro* data raise the possibility that, like the m3-muscarinic receptor, the intracellular kinase mediating receptor phosphorylation in CHO-m1 cells is CK1α.

In a recent study [36], the possibility that GRK-2 may phosphorylate the m1-muscarinic receptor *in vitro* was investigated. Purified m1-muscarinic receptors from Sf9 cells were reconstituted with purified GRK-2 in phospholipid vesicles and, although the receptors were phosphorylated in the basal state, agonist-mediated phosphorylation was observed only after precipitation of the vesicles with poly(ethylene glycol); maximal phosphorylation was observed after 60 min. Taken together with the data presented in the present work, it appears that the m1-muscarinic receptor is able to be phosphorylated *in vitro* by GRK-2 and CK1α in an agonist-dependent fashion, albeit with a relatively slow time course.

Interestingly, the m1-muscarinic receptor purified from Sf9 cells was phosphorylated in the absence of CK1α but to a much lesser extent. Furthermore, this phosphorylation was increased in an agonist-dependent manner, suggesting that a kinase that co-purifies with the receptor is able to mediate agonist-sensitive phosphorylation in a manner similar to that of reconstituted CK1α. Recently, an insect homologue of CK1α has been cloned from *Drosophila melanogaster* [39]. Furthermore, an endogenous muscarinic receptor kinase activity in Sf9 cells that co-purifies with recombinant CK1α has been identified, as measured by phosphorylation of an m3-muscarinic receptor fusion protein [19]. However, the fact that the co-purified kinase does not appear to autophosphorylate and that the fractional enhancement of phosphorylation in the presence of agonist is greater for the co-purified kinase than the exogenously applied CK1α argues against the endogenous kinase being the insect homologue of CK1α. Further experiments are presently underway to address this question.

The identity of the endogenous kinase expressed in CHO cells that is responsible for m1-muscarinic receptor phosphorylation is a difficult and elusive issue. The major problem in this field is the lack of specific inhibitors to potential receptor kinases (including CK1α and the GRKs). In the case of the established receptor-specific kinase, GRK-2, the evidence that the endogenous kinase in cell lines responsible for β_2-adrenergic receptor phosphorylation is GRK-2 has relied on heparin inhibition studies in permeabilized cells [40] and on studies where the dominant-negative mutant of the GRK-2 (GRK2-K220R) has been used [41]. Even using the GRK-2 dominant-negative mutant the data is unclear, since, despite being present in a 15-fold molar excess, GRK2-K220R only inhibited wild-type GRK-2 by 60% in the presence of $\beta\gamma$-subunits [41]. Furthermore, in studies designed to investigate the role of GRK-2 in the phosphorylation of PLC-coupled receptors, the dominant-negative mutant of GRK-2 has been shown to have the capacity to disrupt signalling (in some cases by more than 90% [15]) by a mechanism unconnected to receptor phosphorylation [14,15]. These reports bring into question the use of this reagent in studies addressing PLC-coupled receptor desensitization. Hence, beyond *in vitro* reconstitution studies, experiments designed to determine the endogenous kinases responsible for GPCR phosphorylation and desensitization are difficult and largely equivocal. This appears to be the case, even when these kinases are believed to be the so-called established GPCR kinases such as GRK-2. The data presented in the present work, that CK1α is able to phosphorylate the purified m1-muscarinic receptor, is therefore only indicative of a potential cellular mechanism for m1-muscarinic receptor phosphorylation. It, of course, falls well short of establishing that CK1α is the endogenous kinase in CHO cells which mediates agonist-sensitive phosphorylation. We are presently developing a CK1α dominant-negative mutant and reagents designed to 'knockout' endogenous CK1α activity with the aim of investigating further the role of CK1α in PLC-coupled receptor phosphorylation.

The results of the present study show that m1-muscarinic receptors are able to undergo rapid desensitization via a mechanism that is independent of receptor internalization, PtdIns-(4,5)P_2-pool depletion and Ins(1,4,5)P_3 metabolism, but which involves uncoupling of the receptor from the PLC pathway. Based on the fact that the m1-muscarinic receptor is rapidly phosphorylated following agonist stimulation (possibly by CK1α), this raises the possibility that receptor phosphorylation is responsible solely for desensitization.

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